Kidney International, Vol. 58 (2000), pp. 1902–1911

Characterization of receptors for osteogenic protein-1/bone morphogenetic protein-7 (OP-1/BMP-7) in rat kidneys[1]

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Characterization of receptors for osteogenic protein-1/bone morphogenetic protein-7 (OP-1/BMP-7) in rat kidneys.

Background. Osteogenic protein-1/bone morphogenetic protein-7 (OP-1/BMP-7), a member of the transforming growth factor-β superfamily, has been shown to prevent kidney damage from ischemia/reperfusion injury in rats. The molecular events involved in OP-1 action on kidney are not yet understood.

Methods. In this study, we evaluated the biodistribution of ^{125}I-labeled OP-1 in rat kidneys. Adult rats received a single intravenous injection of 250 μg ^{125}I-labeled OP-1 per kg body wt, a dose that was effective in protecting kidneys from ischemic injury. Tissue localization, in situ hybridization, and immunostaining with a specific receptor antibody were performed to identify OP-1 cellular targets. Also, isolated plasma membranes from kidney cortex and medulla regions were analyzed to identify and characterize receptor structural components that recognize OP-1.

Results. At 10 and 180 minutes following injection, the relative uptake of ^{125}I-labeled OP-1 was consistently higher in kidney cortex than in medulla region. Upon autoradiography, kidney tissue sections revealed that OP-1 bound to the convoluted tubule epithelium, glomeruli, and collecting ducts. Moreover, in situ hybridization and immunostaining methods have shown localization of mRNA transcripts and the protein for BMP receptor type II in the cortex and medulla in similar areas as ^{125}I-labeled OP-1. Bulk membranes (enriched with plasma membranes) isolated from the cortex and medulla regions of kidney each bound specifically to ^{125}I-OP-1, and the binding of ^{125}I-labeled OP-1 was inhibited by unlabeled OP-1 in a dose-dependent manner. However, platelet-derived growth factor, transforming growth factor-β, insulin-like growth factor, fibroblast growth factors, and other members of BMP family such as BMP-2 and cartilage-derived morphogenetic protein-1/growth and differentiation factor-5 (CDMP-1/GDF-5) failed to inhibit the binding of ^{125}I-labeled OP-1 to receptors, suggesting a high degree of specificity with which OP-1 bound to kidney receptors. Scatchard analysis of quantitative binding data indicated that the OP-1 receptors of kidney contained a single class of high-affinity binding sites for OP-1 with an association constant (K_a) of 2.26×10^9 mol/L^{-1} and a binding capacity of 1.01 pmol of OP-1 per mg membrane protein. When analyzed by a ligand blot technique, plasma membranes isolated from kidney cortex and medulla each showed the presence of a prominent specific band with a relative molecular mass (M_r) of 100 kD. Further analysis by Western blotting indicated that an antibody raised against BMP type II receptor effectively recognized the 100 kD OP-1 binding component of kidney plasma membranes.

Conclusions. We demonstrated, to our knowledge for the first time, the presence of membrane-bound, specific, high-affinity OP-1 receptors in rat kidney tissues, which are likely to mediate OP-1 actions in the kidney. The major OP-1–binding component of the kidney appears to be a long form of BMP type II receptor with a M_r of 100 kD. In vivo and in vitro evidence suggests that the cellular targets for OP-1 are convoluted tubule epithelium, glomeruli, and collecting ducts. OP-1 does not share receptor binding properties with other growth factors, including BMP-2 and CDMP-1, suggesting that its mode of action in kidney appears to be specific.

Bone morphogenetic proteins (BMPs) are a family of multifunctional proteins originally identified as proteins that induce bone and cartilage formation at ectopic extraskeletal sites in vivo [1, 2]. These are glycosylated proteins and belong to the transforming growth factor-β (TGF-β) superfamily. BMP-2 and BMP-4 are highly similar to each other in structure (92% amino acid sequence identity), while they are distantly related to osteogenic protein-1 (OP-1/BMP-7; 58 to 60% amino acid sequence identity). In vitro, OP-1 stimulates alkaline phosphatase activity and collagen synthesis in osteoblasts, proteoglycan synthesis in chondroblasts, chemotaxis of monocytes, and differentiation of neural cells [3, 4]. Also, OP-1 has important roles in the morphogenesis of embryo and in postnatal life [5, 6]. It is produced in the kidney [5, 7], induces nephrogenesis, and is required for eye development and skeletal patterning [8–10]. More recent studies have shown that OP-1 is able to prevent kidney damage

[1] **See Editorial by Kopp, p. 2237**

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Key words: transforming growth factor-β superfamily, convoluted tubule epithelium, glomeruli, receptor binding, ischemia/reperfusion injury.

Received for publication July 14, 1999
and in revised form May 25, 2000
Accepted for publication May 30, 2000

from ischemia/reperfusion injury in rats [11]. The molecular events involved in OP-1's action on the kidney are not yet understood. In this study, we evaluated the biodistribution of ^{125}I-labeled OP-1 in the rat kidney and analyzed kidney bulk membranes to identify and characterize the OP-1 receptor structural component(s) that recognizes OP-1.

METHODS

Preparation of ^{125}I-labeled OP-1

Highly purified mature OP-1 (15.7 mg) was radioiodinated with 5 mCi of carrier-free Na^{125}I using a modification of the lactoperoxidase method of Dattatreyamurty et al [12]. Gel filtration on a Sephadex G-25 column was used to separate radioiodinated OP-1 from free iodide. The column was eluted with 20 mmol/L sodium acetate buffer, pH 4.5, containing 0.2% Tween-80 and 0.1% ovalbumin. Specific activity of the radioiodinated OP-1 preparation used in this study was 0.237 μCi/μg.

Biodistribution of ^{125}I-labeled OP-1

Sprague-Dawley rats with an average body weight of 400 g received a single intravenous injection of ^{125}I-labeled OP-1 at a dose level of 250 or 1000 μg/kg body weight. Animals were sacrificed at 10 or 180 minutes following injection. One kidney and liver were removed, fixed in 4% paraformaldehyde, and postfixed in 70% ethanol. The other kidney (unfixed) was dissected to separate cortex and medulla. The relative uptake of ^{125}I-labeled OP-1 by tissues was expressed as ng of radiolabeled OP-1 per gram wet tissue weight. To evaluate tissue distribution of radioiodinated OP-1, the kidney sections were coated with Kodak NTB2 emulsion, exposed for two weeks at 4°C, and lightly stained with Gienesa stain as previously described [13].

In situ hybridization

Bone morphogenetic protein receptor type II mRNA distribution was analyzed on kidney sections using specific RNA sense and antisense probes (a gift from Peter ten Dijke) [14], and in situ hybridization was performed as previously described [5].

Preparation of plasma membrane-enriched fractions (bulk membranes) from rat kidney cortex and medulla

Kidneys from normal young rats were dissected to separate cortex and medulla. The tissues were separately homogenized, and membranes were isolated using a modification of the procedure described earlier [15]. Although these membranes are enriched with plasma membranes, as revealed by the content of a marker enzyme, 5′-nucleotidase, they were not purified further and therefore should be considered as bulk membranes. Membranes were suspended in 50 mmol/L HEPES buffer, pH 7.4, containing 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 0.1 mol/L sucrose at a protein concentration of 11.5 mg/mL, and they were snap frozen and stored at −70°C until used in the binding assay.

Specific uptake of ^{125}I-labeled OP-1 by rat kidney membrane receptor

Highly purified soluble OP-1 (25 μg) was radioiodinated to a high specific activity (70 to 76 μCi/μg) with 2 mCi of carrier-free Na^{125}I, using a modification of the lactoperoxidase method of Dattatreyamurty, Schneyer and Reichert [12]. Bulk membranes isolated from rat kidney cortex and medulla were separately incubated with approximately 300,000 cpm (3 ng) of ^{125}I-labeled OP-1 in the absence or presence of excess unlabeled OP-1. Membrane (receptor)-bound radioligand was separated from free radioligand by centrifugation at 30,000 × *g* for 60 minutes at 4°C. The final pellet containing receptor-bound ^{125}I-labeled OP-1 was counted in an auto-γ counter. Values reflecting specific binding are expressed as a percentage of total radioligand added to the membrane-bound receptor (B/T%).

Radioligand receptor binding assay

Using bulk membranes of rat kidney cortex as the source for receptor and ^{125}I-labeled OP-1 as a ligand, we developed a sensitive radioligand receptor binding assay. The assay was carried out as follows: 150 μL of assay buffer [50 mmol/L HEPES, pH 7.4, containing 10 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.1 mol/L sucrose, and 1% bovine serum albumin (BSA)] or assay buffer containing increasing concentrations of unlabeled OP-1 (5 ng to 10.48 μg) was added to assay tubes. Membranes (approximately 200 μg protein in 200 μL of 50 mmol/L HEPES buffer, pH 7.4, containing 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 0.1 mol/L sucrose) and approximately 300,000 cpm (3 ng/50 μL of assay buffer) of ^{125}I-labeled OP-1 were added to assay tubes and were incubated for 22 hours while rocking at 4°C. To the tubes, 400 μL of HEPES buffer without sucrose were added. The contents were mixed and centrifuged at 30,000 × *g* for 45 minutes at 4°C to separate free radioligand from receptor-bound radioligand. The supernatant was aspirated, and pellet was suspended in 0.8 mL HEPES buffer without sucrose. The tubes were then centrifuged at 30,000 × *g* for 45 minutes at 4°C. The supernatant was aspirated, and the radioactivity of the pellet containing receptor-bound radioligand was counted in an auto-γ counter. The results (B/B_0%) are expressed as a percentage of specific binding determined in the absence of unlabeled OP-1. The affinity constant (K_a) was determined from competitive data by Scatchard analysis using the LIGAND program of Munson and Rodbard [16].

To evaluate the specificity of OP-1 interaction with the kidney receptors, we performed a radioligand receptor

assay. In this assay, kidney cortex bulk membranes were incubated for 20 hours with 300,000 cpm (approximately 3 ng) of ^{125}I-labeled OP-1 in the presence or absence of increasing concentrations of unlabeled OP-1, platelet-derived growth factor (PDGF), TGF-β, insulin-like growth factor (IGF), fibroblast growth factor (FGF), cartilage-derived morphogenetic protein (CDMP), and BMP-2 (all prepared at Creative BioMolecules) for 20 hours at 4°C. Radioligand receptor binding assay was performed essentially as described previously in this article. The results (B/B_0%) are expressed as a percentage of specific binding determined in the absence of unlabeled OP-1 or other growth factors.

Ligand blot analysis of the OP-1 receptor in rat kidney bulk membranes

Membranes isolated from rat kidney cortex or medulla were treated with sodium dodecyl sulfate (SDS; final concentration 1.6%, wt/vol) in the presence of 14.3% glycerol, without prior heating, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 5 to 9% acrylamide gradient gels under nonreducing conditions according to the procedure of Laemmli [17]. Electrophoresis of samples was carried out at 35 mA constant current at 4°C. Resolved proteins were transblotted onto polyvinylidene difluoride (PVDF) membranes using a Pharmacia-LKB 2005 Transphor unit at 0.2 A and 4°C for 16 hours. The sample lanes were incubated with blocking buffer (3% BSA in 50 mmol/L HEPES, pH 7.4, containing 5 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$) overnight at 4°C and were further incubated for 22 hours in HEPES buffer containing 0.5% BSA, 10 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and ^{125}I-labeled OP-1 ($\sim 8 \times 10^5$ cpm/mL) in the absence or presence of excess unlabeled OP-1. The blots were rinsed with 50 mmol/L HEPES buffer containing 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, pH 7.4, and were dried and subjected to autoradiography. The position of the M_r standards is indicated on the left side of sample lines. The position of specific bands indicating OP-1 receptor is shown by an arrow in Figure 7.

Western blot analysis of OP-1 receptors in rat kidney bulk membranes

A rabbit antiserum raised against the N-terminus peptide region (Ser[185]-Asn[202]) in the extracellular domain of type II BMP receptor was affinity purified to isolate immune IgG. The site-directed antibody (50 μg) was radioiodinated with 1.5 mCi of Na^{125}I (carrier free) by using the chloramine-T method. As described in the ligand blot analysis, kidney bulk membranes were treated with SDS and were subjected to SDS-PAGE, and then sample proteins were transblotted onto PVDF membranes. The sample lanes were incubated with blocking buffer (2.5% BSA in 50 mmol/L Tris-HCl buffer, pH 7.4, containing 150 mmol/L NaCl) overnight at 4°C and further incubated for 20 hours at room temperature in blocking buffer containing ^{125}I-labeled anti-BMP type II receptor antibody ($\sim 5 \times 10^5$ cpm/mL). The blots were washed four times with 50 mmol/L Tris-HCl buffer containing 150 mmol/L NaCl and 0.05% Tween-20, dried and subjected to autoradiography. The position of the M_r standards is indicated on the left side of sample lines. The position of specific bands indicating OP-1 receptor is shown by an arrow (Fig. 7).

RESULTS

Biodistribution of ^{125}I-labeled OP-1 in rat kidneys and liver

Sprague-Dawley rats were injected intravenously with ^{125}I-labeled OP-1 at dose levels of 250 μg and 1000 μg/kg body weight. The corresponding doses of the low specific activity ^{125}I-labeled OP-1 preparation were 59.26 μCi and 237 μCi/kg body weight, respectively. Animals were sacrificed at intervals of 10 minutes and 3 hours following injections. Blood levels of ^{125}I-labeled OP-1 decreased rapidly over first 15 minutes following injection, and thereafter, the levels declined slowly. Following OP-1 injection at a low dose, the average OP-1 uptakes by kidney cortex observed at 10 and 180 minutes were 270 and 80 ng/g tissue, respectively (Fig. 1A). At a higher OP-1 dose level, the distribution of OP-1 in kidney cortex did not show much of an increase (Fig. 1B). Interestingly, the relative uptake of OP-1 observed at 10 and 180 minutes following intravenous injection was consistently higher in the kidney cortex than in the medulla (Fig. 1 A, B). Also, a higher distribution of ^{125}I-labeled OP-1 was observed in the liver.

Cellular localization of ^{125}I-labeled OP-1 and BMP receptor type II in rat kidney

Following an intravenous injection of ^{125}I-labeled OP-1, kidneys were processed further to evaluate the cellular distribution of radioiodinated OP-1. Upon autoradiography, kidney sections revealed that the majority of ^{125}I-labeled OP-1 was accumulated in glomeruli and adjacent convoluted tubules, while in the medulla, ^{125}I-labeled OP-1 grains accumulated in the collecting ducts at 10 and 180 minutes following injection (Fig. 2 A–F). In situ hybridization and immunostaining experiments demonstrated that both mRNA transcripts and the protein for BMP receptor type II were localized in the cortex and medulla in similar areas as ^{125}I-labeled OP-1 (Figs. 2 G, H and 3 A–C).

Identification of specific receptors for OP-1 in rat kidney plasma membranes

Membranes were isolated from the cortex and medulla of rat kidneys and were analyzed for specific binding of OP-1 by a radioligand receptor binding assay. When sev-

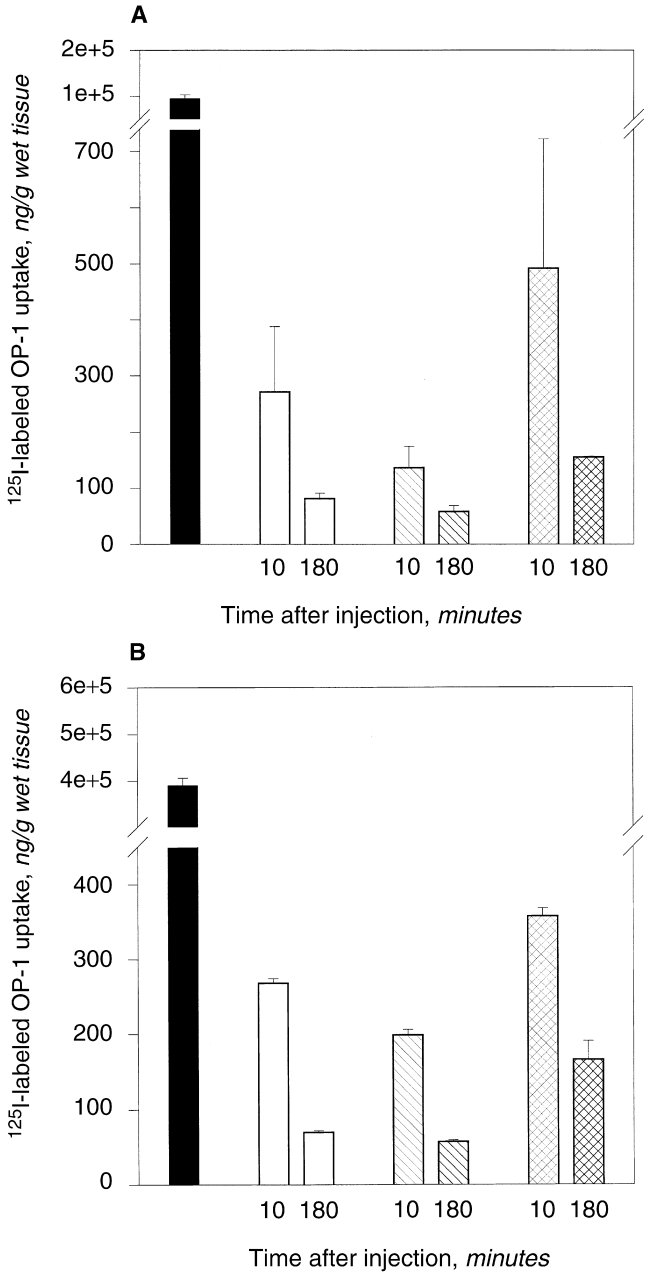


Fig. 1. Biodistribution of ^{125}I-labeled osteogenic protein-1 (OP-1) in rat kidney and liver. Sprague-Dawley rats received a single intravenous injection of ^{125}I-labeled OP-1 at two different dose levels: (*A*) 250 μg/k · g · body wt; (*B*) 1000 μg/kg body weight. Animals were sacrificed at 10 or 180 minutes following injection. Rats were perfused with saline and dissected to separate cortex from medulla. The relative uptake of ^{125}I-labeled OP-1 by tissues was expressed as ng of radiolabeled OP-1 per gram wet tissue weight. Symbols are: (■) injected dose; (□) kidney cortex; (▨) kidney medulla; (⊠) liver.

eral batches of membranes isolated from kidney cortex and medulla were separately incubated with ^{125}I-labeled OP-1 (~3 ng) for 20 hours at 4°C, a significant uptake of radioiodinated OP-1 by each batch of membranes was observed. In the presence of excess unlabeled OP-1, the binding of radioiodinated OP-1 to membranes, however, decreased markedly because of inhibition by unlabeled OP-1. These results thus provide an initial evidence for the presence of OP-1 binding sites in kidney membranes. Moreover, the relative OP-1 binding (per mg tissue protein) was observed to be higher in the kidney cortex than in the medulla (Fig. 4).

Affinity and binding capacity of OP-1 receptors in kidney cortex

Experiments were carried out to understand the characteristics of OP-1 binding to receptors in the kidney cortex. Initially, uptake of ^{125}I-labeled OP-1 by increasing amounts of plasma membranes of kidney cortex was examined. From the OP-1 uptake profile, optimum concentrations of ligand and plasma membrane protein were determined to set up a sensitive radioligand receptor assay. In this assay, the binding of ^{125}I-labeled OP-1 to the kidney cortex plasma membranes was inhibited by unlabeled OP-1 in a dose-dependent manner (Fig. 5A). From the quantitative competitive binding data, the K_a and the binding capacity of OP-1 receptors in the kidney cortex were determined by Scatchard analysis using the LIGAND program. The results indicated that the OP-1 receptors of kidney cortex plasma membranes contained a single class of high-affinity binding sites for OP-1 with a K_a of 2.26×10^9 mol/L^{-1} and the binding capacity of 1.01 pmol of OP-1 per mg membrane protein (Fig. 5B). The calculated binding capacity of receptors per gram tissue of kidney cortex was 39.7 pmol OP-1.

Specificity of OP-1 binding to kidney cortex

Radioligand receptor assay was carried out to examine the specificity with which OP-1 binds to kidney cortex receptors. In these assays, the effects of increasing concentrations of unlabeled OP-1, PDGF, TGF-β, IGF, and FGF on the binding of ^{125}I-labeled OP-1 to receptor-enriched plasma membranes of kidney cortex were examined. Unlabeled OP-1 gave a dose-dependent linear inhibitory response, while other growth factors such as PDGF, TGF-β, IGF, and FGF failed to inhibit the binding of ^{125}I-labeled OP-1 to kidney cortex plasma membranes (Fig. 6). These results thus suggest that OP-1 interacts with kidney cortex receptors with a high degree of specificity. Experiments were continued to examine whether other members of BMP family affect OP-1 binding to the kidney cortex. Two purified preparations, BMP-2 and CDMP-1, were tested in a radioligand receptor assay. In this assay, BMP-2 and CDMP-1 failed to inhibit the binding of ^{125}I-labeled OP-1 to rat kidney cortex plasma membranes, while unlabeled OP-1 gave a dose-dependent inhibition curve (Fig. 6B). These results further substantiate that the OP-1 interaction with the kidney cortex receptor is highly specific and is not shared by BMP-2 and CDMP-1.

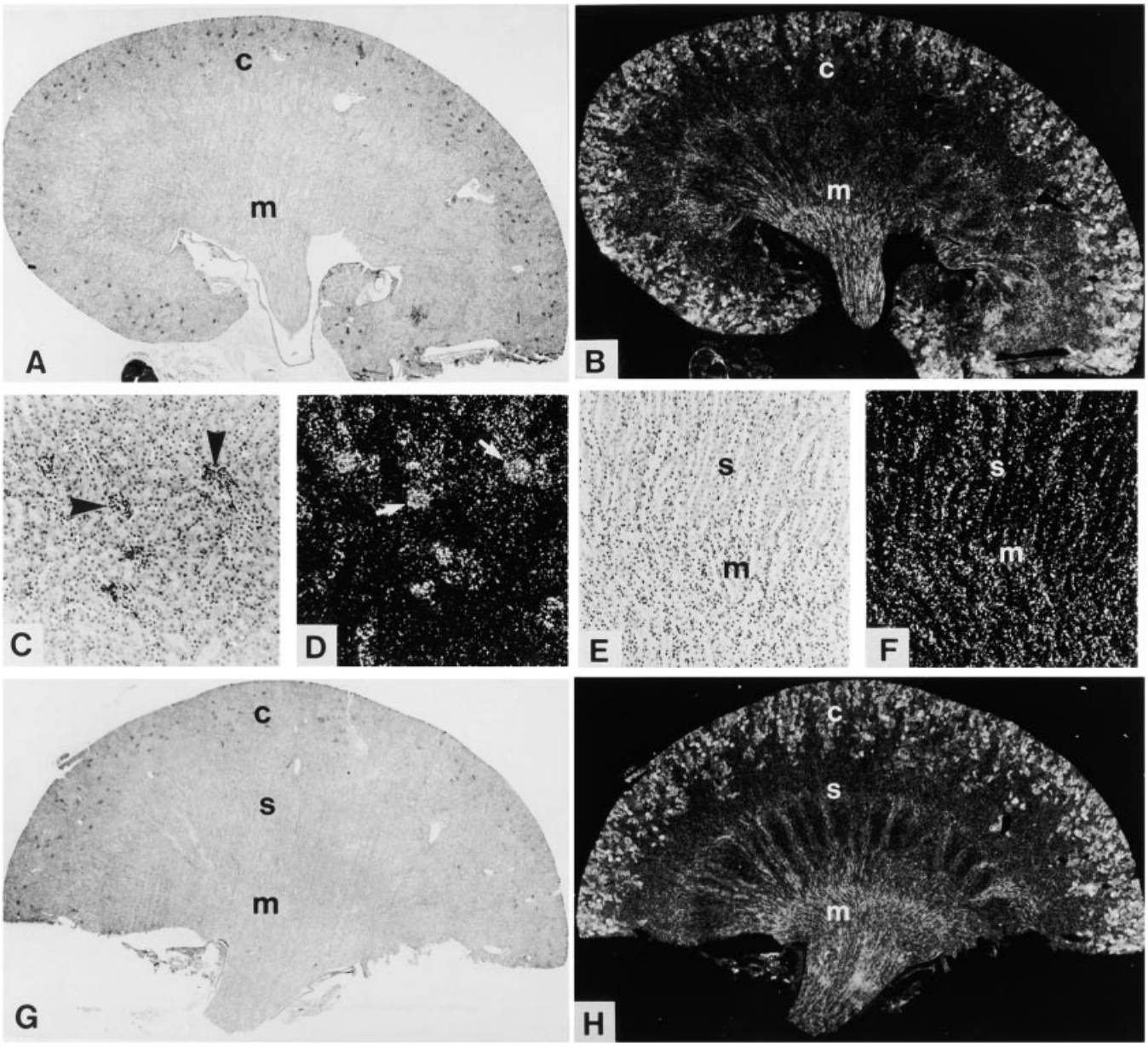
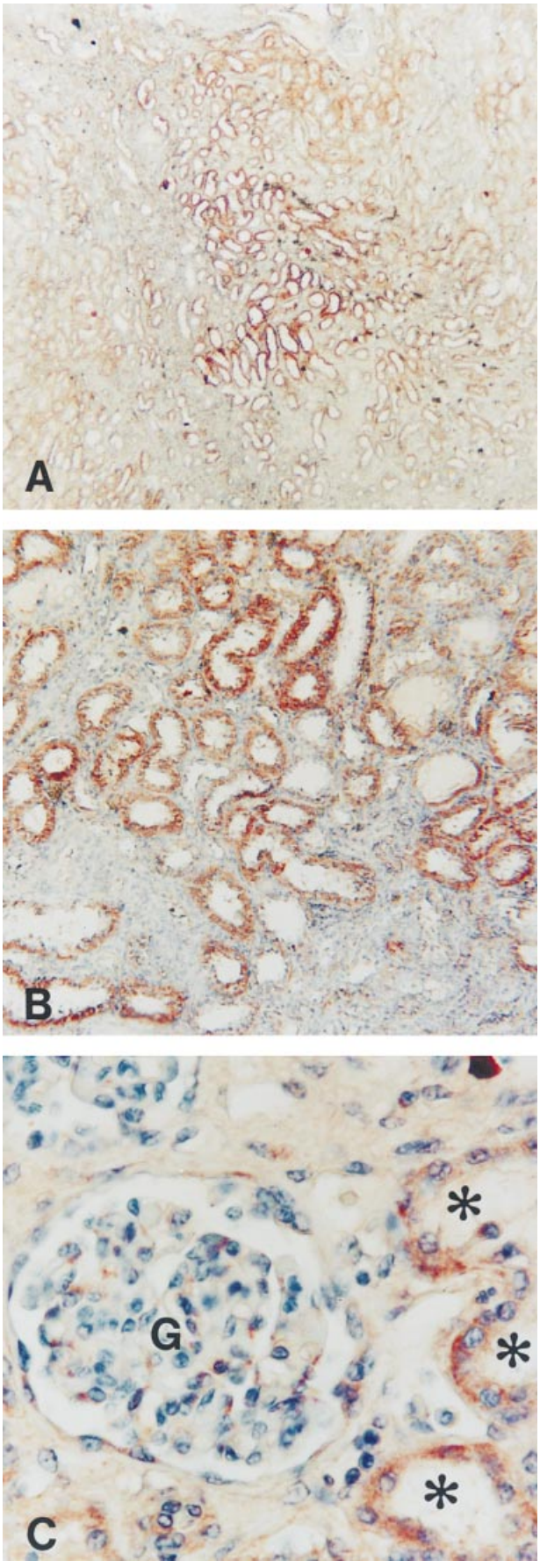


Fig. 2. Localization of ^{125}I-labeled osteogenic protein-1 (OP-1) and bone morphogenetic protein (BMP) type II receptor mRNA in rat kidney. Rats received a single intravenous injection of 250 μg/kg body weight and were killed at 10 minutes following injection (*A–F*). (A) Hematoxylin and eosin-stained brightfield image of a kidney midsagittal section (c, cortex; m, medulla). (B) Darkfield image of the same section with ^{125}I-labeled OP-1 accumulated in the cortex. (C) Enlarged cortex area from A showing glomeruli (arrowheads) and adjacent convoluted tubules. (D) Darkfield image of C, indicating accumulation of grains in glomeruli (arrows) and adjacent convoluted tubules. (E) Enlarged medulla (m) and S3 zone (s) from A. (F) Darkfield image of E, indicating accumulation of grains in epithelium of collecting ducts. For in situ hybridization, rat kidney sections of 5 to 7 μm thick were cut, mounted on silinated slides, and hybridized with a BMP receptor type II probe for 18 hours at 50°C in 50% formamide, as previously described [5]. (*G*) Toluidine blue-stained brightfield image of a kidney midsagittal section. Abbreviations are: c, cortex; s, S3 zone; m, medulla. (*H*) Darkfield image of an adjacent section with mRNA transcript localized in the cortex and medulla in a similar pattern as ^{125}I-labeled OP-1 (magnification ×5 in A, B, G, and H, and ×200 in C–F).

Identification of type II receptors in kidney plasma membranes

A method (the ligand blot technique) was developed and used to identify OP-1 receptor structural components in plasma membranes of rat kidneys. The advantage of this method over a previously employed covalent cross-linking approach is that it allows direct identification of active receptor as well as measurement of relative molecular mass (M_r) of the receptor proper, but not of the receptor-ligand complex. As the radiolabeled OP-1 binding to plasma membranes of medulla was relatively lower than that of plasma membranes of cortex, the mem-



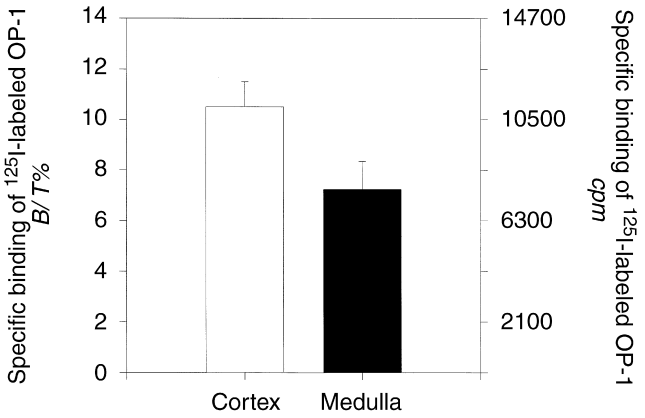


Fig. 4. Specific uptake of ^{125}I-labeled OP-1 by rat kidney bulk membrane receptors. Membranes isolated from rat kidney cortex and medulla were incubated with 300,000 cpm (approximately 3 ng) of ^{125}I-labeled OP-1 in the absence or presence of excess unlabeled OP-1. Radioligand assay was performed as described in the **Methods** section. Values reflecting specific binding of ^{125}I-labeled OP-1 are expressed as a percentage of total radioligand added to the membrane-bound receptor (B/T%). Each point is the average of triplicate determinations.

branes from medulla were analyzed at a higher protein concentration (1.6-fold). When both of the membranes isolated from kidney cortex or medulla were analyzed by ligand blotting, each showed the presence of a prominent band. This band, however, disappeared when the sample-containing blots were incubated with ^{125}I-labeled OP-1 in the presence of excess unlabeled OP-1, suggesting a high degree of specificity with which OP-1 bound to this component. A calibration plot of relative mobility (R_f) versus log molecular weights of protein markers was established. The M_r of the identified OP-1 binding component, as determined from the plot, was 100 kD (Fig. 7A).

Recently, Rosenzweig et al cloned the type II receptor for BMPs and expressed it in COS-1 cells [14]. The recombinant type II receptor is much larger than type I receptors, and it has an M_r of approximately 100 kD. Since both kidney cortex and medulla appeared to contain an OP-1–binding component with similar M_r, we further examined the identity of this component by developing a Western blot method using ^{125}I-labeled anti-BMP type II receptor antibody. This is a polyclonal antibody raised against a synthetic peptide corresponding to a variable region (Ser185-Asn202) in the extracellular domain of BMP receptor type II. The radioiodinated antireceptor antibody effectively recognized the 100 kD

Fig. 3. Immunostaining of a rat kidney with a BMP receptor type II polyclonal antibody. Strong positive staining was observed in convoluted tubules and glomeruli (*A* and *B*). At higher magnification (*C*), BMP receptor type II protein is present in both a glomerulus (G) and proximal tubules (magnification ×25 in A, ×100 in B, and ×400 in C).

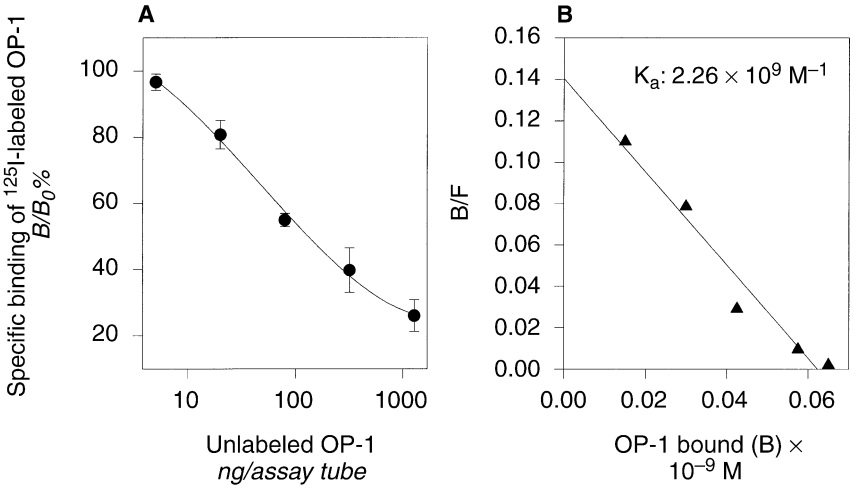


Fig. 5. (*A*) Effect of increasing concentrations of unlabeled OP-1 (dose–response curve) on the uptake of ^{125}I-labeled OP-1 by kidney membrane-bound receptors. ^{125}I-labeled OP-1 was incubated in the absence or presence of indicated concentrations of unlabeled OP-1 with kidney membranes (~200 μg protein) for 22 hours in cold (4°C). Radioligand receptor assay was essentially carried out as described in the **Methods** section. Values (B/B_0%) are expressed as a percentage of specific binding determined in the absence of unlabeled OP-1. Each point is the average of triplicate determinations. (*B*) Scatchard plot for the binding of ^{125}I-labeled OP-1 to receptors in kidney bulk membranes. Affinity constant (K_a) was determined from competitive data by Scatchard analysis using the LIGAND program of Munson and Rodbard [16].

OP-1 binding component of rat kidney membranes (Fig. 7B). The observed band, however, disappeared when the blot was incubated with the radiolabeled antibody that was preincubated with the immunizing receptor peptide (data not shown). These results indicate that the identified 100 kD component is possibly BMP type II receptor, which is consistent with the localization of BMP receptor type II in the cortex and medulla, as shown by in situ hybridization and immunostaining methods (Figs. 2 and 3).

DISCUSSION

It has been well established that the in vivo efficacy of drug candidates depends not only on their activity profiles, but also on their ability to target specific tissues. Targeting of a given drug is better understood by its bioavailability and cellular distribution in tissues. Our study represents the first examination, to our knowledge, of OP-1 targeting to kidneys after systemic administration in rats. Rats received a single intravenous injection of ^{125}I-labeled OP-1 at an effective dose of 250 μg/kg body weight or a dose four times higher. Radioactive OP-1 cleared in a biphasic manner, with a rapid clearance over the initial 15 minutes and substantially slower clearance thereafter. We observed an uptake of labeled OP-1 in both the cortex and medulla regions of kidney. The relative uptake of radiolabeled OP-1, however, was higher in the cortex. The average OP-1 uptake by kidney cortex observed at 10 and 180 minutes was 270 ng and 80 ng/g tissue, respectively. These values of OP-1 are not considered to be low, since studies with other growth factors such as TGF-β and activin also showed low tissue distribution [18, 19]. It has been shown that OP-1 at these concentrations is effective in cell cultures in maintaining epithelial phenotype of human proximal epithelial cells. Increasing the total injected dose of OP-1, however, had no added advantage, as the relative uptake of OP-1 by the kidney did not substantially increase, suggesting that the OP-1 binding sites in kidneys have already reached close to a saturation level at the lower OP-1 dose tested. Interestingly, tissue autoradiography, in situ hybridization, and immunostaining with a site-directed receptor antibody all identified the convoluted tubule epithelium and glomeruli in the cortex and the collecting ducts of medulla as the cellular targets for OP-1. Previous studies have shown that the rat kidney is the major source for OP-1 and that the major site of OP-1 production is the epithelium of the collecting ducts within the medulla (abstract; Jin et al, *2nd International Conference on Bone Morphogenetic Proteins*, Sacramento, A96, 1997) [20]. Taken together, these results suggest that OP-1 might have both paracrine and autocrine roles in the kidney.

It is pertinent to mention that tissue autoradiography has shown localization of radiolabeled OP-1 in the S3 segment. Moreover, by in situ hybridization, it has been found that epithelial cells in the S3 zone synthesize OP-1 mRNA. Therefore, it is likely that in case of an ischemic injury within the S3 zone, exogenously administered OP-1 binds to cell receptors and protects from necrosis and infarction, as has been previously demonstrated [11].

A higher uptake of ^{125}I-labeled OP-1 by liver observed in this study cannot be attributed to a nonspecific binding, as it has been shown previously that liver has specific BMP receptors [21]. Alternatively, this may reflect an indirect binding of OP-1 to the liver. The indirect binding of OP-1 may be facilitated by a binding protein present in circulation. Recently, we have shown that α2-macroglobulin, which is present in high concentrations in blood, is the predominant binding protein for OP-1 (abstract; Dattatreyamurty, *2nd International Conference on Bone Morphogenetic Proteins*, Sacramento, A126, 1997). It is important to note that upon activation by protease, α2-macroglobulin undergoes a conformational change that

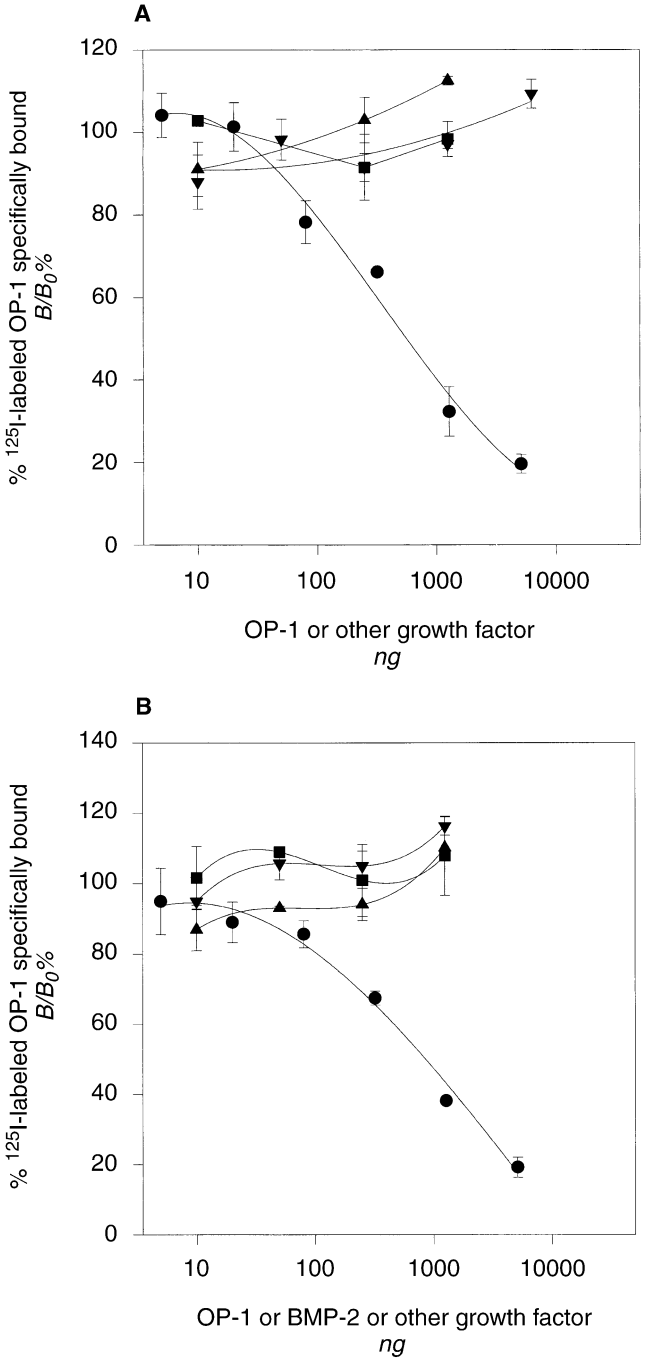


Fig. 6. Effect of other growth factors and unlabeled BMPs on the binding of ^{125}I-labeled OP-1 to receptor-enriched kidney cortex bulk membranes. Membranes were incubated with 300,000 cpm (~3 ng) of ^{125}I-labeled OP-1 in the presence or absence of increasing concentrations of (*A*) unlabeled OP-1 (●), PDGF (■), TGF-β (▲), and IGF (▼), or (*B*) unlabeled OP-1 (●), FGF (■), CDMP (▼), and BMP-2 (▲) for 20 hours at 4°C. Radioligand assay was performed as described in the **Methods** section. Values (B/B_0%) are expressed as a percentage of specific binding determined in the absence of unlabeled OP-1 or other growth factors. Each point is the average of triplicate determinations.

exposes a previously buried domain close to the carboxyl terminus that is then recognized by a cell surface receptor system in the liver, which mediates binding and endocytosis of the complex. Recent studies have shown that this is the mechanism by which TGF-β is targeted to liver by binding to activated α2-macroglobulin [22]. In an independent study, we have shown that OP-1 bound to native as well as activated forms of α2-macroglobulin with similar affinities (unpublished data). These results thus could partly explain why exogenously administered OP-1 accumulated in the liver. Further experiments, however, are required to address these possibilities.

It is noteworthy that plasma membranes from both kidney cortex and medulla showed the presence of specific receptors for OP-1. The relative abundance of OP-1 binding sites in cortex membranes was much higher than in medulla region. Moreover, Scatchard analysis indicated that the receptors in kidney cortex contained a single class of high-affinity OP-1 binding sites, with a K_a of 2.26×10^9 mol/L^{-1}. The calculated binding capacity of receptors per mg membrane protein was 1.01 pmol OP-1. In a previous study, a closely related growth factor, TGF-β, and its interaction with the receptors were studied in the proximal tubules isolated from rabbit renal cortex [23]. Interestingly, the binding data revealed the presence of both high- and low-affinity binding sites for TGF-β in the renal cortex. Our study, however, showed no evidence of low-affinity OP-1 binding sites in all batches of kidney cortex plasma membranes tested. It is important to note that the endogenous levels of TGF-β and other related growth factors are normally low, and high-affinity and low-capacity receptors for these factors are implicated to mediate their actions.

Another noteworthy observation is the high degree of specificity with which OP-1 interacts with the kidney receptors. Thus, other growth factors such as PDGF, TGF-β, IGF, and FGF, even at high concentrations, failed to inhibit the binding of ^{125}I-labeled OP-1 to kidney plasma membrane receptors. Similarly, other members of BMP family such as BMP-2 and CDMP-1 also failed to affect OP-1's interaction with kidney receptors. These results clearly indicate that OP-1 does not share receptor-binding properties with the other growth factors examined, and its mode of action in the kidney appears to be specific. It is important to note that BMP-2 and CDMP-1 show only 60 and 51% homology, respectively, with the primary sequence of OP-1, suggesting that an OP-1 interaction with kidney cortex receptors may involve regions in OP-1 that are not well conserved among these growth factors.

Recently, Miyazono and his associates cloned type I and type II receptors for BMPs and expressed them in COS cells [14, 24]. OP-1 was shown to bind to two recombinant type I receptors, ALK-2 and ALK-6, and to ALK-3 less efficiently, and these ALK receptors had

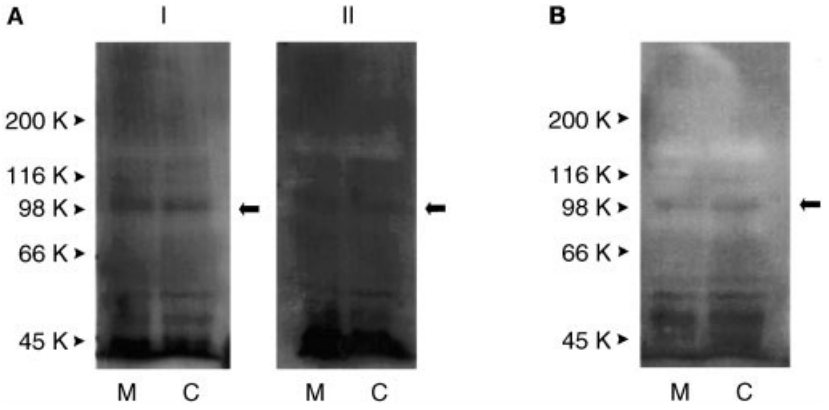


Fig. 7. Ligand blot or Western blot analysis of the OP-1 receptor in rat kidney bulk membranes. Membranes isolated from rat kidney cortex (C) or medulla (M) were treated with SDS and subjected to SDS-PAGE in 5 to 9% acrylamide gradient gels under nonreducing conditions and without prior heating of the samples. After electrophoresis, resolved sample proteins were transblotted onto PVDF membranes. The sample-containing blots were further subjected to either ligand blot or Western blot analysis. (*A*) Ligand-blotting: The sample lanes were incubated in blocking buffer overnight at 4°C and then for 22 hours at 4°C in HEPES buffer containing 0.5% BSA, 10 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and ^{125}I-labeled OP-1 ($\sim 8 \times 10^5$ cpm/mL) in the absence (I) or presence (II) of excess unlabeled OP-1. The blots were rinsed with 50 mmol/L HEPES buffer containing 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, pH 7.4, and were dried and subjected to autoradiography. (*B*) Western blotting: The sample lanes were incubated in blocking buffer (2.5% BSA in 50 mmol/L Tris-HCl buffer, pH 7.4, containing 150 mmol/L NaCl) overnight at 4°C and then for 20 hours at room temperature in blocking buffer containing ^{125}I-labeled anti-BMP type II receptor antibody (approximately 5×10^5 cpm/mL). The blots were washed four times with 50 mmol/L Tris-HCl buffer containing 150 mmol/L NaCl and 0.05% Tween-20 and were dried and subjected to autoradiography. The position of the M_r standards are indicated on the left side of sample lines. The position of specific band indicating OP-1 receptor is shown by an arrow.

M_r values in the range of 50 to 58 kD. On the other hand, the recombinant type II receptor is much larger, and it has two forms, a truncated form with no C-terminus extension [25] and a long form with an M_r of approximately 100 kD [14]. The type II receptor can effectively bind OP-1 on its own, while type I receptors are required to be coexpressed with type II receptor for efficient binding to OP-1. In the present study, when plasma membranes isolated from kidney cortex or medulla were analyzed by ligand blotting, each showed the presence of a prominent band with an M_r of 100 kD. Interestingly, the size of the OP-1 binding component of rat kidney appears to match with the M_r of cloned BMP type II receptor. Further analysis by Western blot method using a site-directed receptor antibody identified the 100 kD component possibly as a BMP type II receptor. Consistent with this observation, both in situ hybridization and immunostaining methods have shown that mRNA transcripts and the protein for BMP receptor type II are localized in glomeruli and adjacent convoluted tubules of cortex, and in the collecting ducts of medulla.

Bone morphogenetic proteins, TGF-β, IGF-I, and IGF-II are recognized as signaling molecules that control growth and differentiation during embryogenesis. Recently, it has been shown that OP-1 is capable of protecting the kidney against acute renal failure in postnatal life [11]. Garcia-Ocana, Penaranda, and Esbrit have shown that hypertrophy of the proximal tubule is associated with an increased production of both TGF-β as well as TGF-β receptors [23]. On the other hand, in experimental membranous nephropathy, injury to glomerular epithelial cells is associated with an up-regulation of the TGF-β2 and TGF-β3 isoforms, and an increase in TGF-β type I and type II receptor expression. Studies by Flyvbjerg et al have shown that an initial increase in renal size and function in experimental diabetic kidney is always preceded by an increase in renal IGF-I, IGF-binding proteins, and IGF receptor concentrations [26]. Clearly, those and our present studies signify the importance of OP-1, OP-1 receptor, TGF-β, TGF receptors, IGF and IGF receptors as major regulators in kidney physiology and renal repair. Whether OP-1 receptors in renal proximal tubules and glomeruli show similar concentration changes to regulate tubular cell growth and differentiation after renal injury remains to be elucidated.

In conclusion, to our knowledge for the first time, we have demonstrated the presence of membrane-bound, specific, high-affinity OP-1 receptors in rat kidney tissues to mediate OP-1 actions. The major OP-1–binding component of kidney may be a long form of BMP type II receptor with an M_r of 100 kD. In vivo evidence suggests that the cellular target for OP-1 are the convoluted tubule epithelium and the glomeruli in the cortex, and the collecting ducts in the medulla region. Moreover, in situ hybridization and immunostaining methods have shown localization of mRNA transcripts and the protein for BMP receptor type II in similar areas of cortex and medulla.

Osteogenic protein-1 does not share receptor-binding properties with other growth factors, including BMP-2

and CDMP-1, suggesting that its mode of action in kidney appears to be specific. These findings provide a molecular basis for the interaction of OP-1 with different kidney regions.

ACKNOWLEDGMENTS

The authors acknowledge the gift of antiserum to BMP type II receptor (raised against peptide region, Ser185-Asn202) provided by Dr. Peter ten Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden. We thank D. Drager for excellent technical help in biodistribution studies.

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